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The Molecular Chaperone Skp Reduces Client Protein Aggregation Under *In Vivo-like* Conditions

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The Molecular Chaperone Skp Reduces Client Protein Aggregation Under *In Vivo-like* Conditions

by

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Dedication

This thesis is dedicated to:

Grandma, Papo, Mom, Dad, Sarah, Abby, Chris, Kelly and Chris

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Truly extraordinary people surround me. This thesis is a product the hard work of many people beside myself. My research advisor, Dr. Jennifer A. Maynard, not only is a powerhouse academic scientist with patents for antibody therapeutics, but on top of that she engages students at all levels in scientific inquiry. I had a very naïve understanding of how scientific evidence is constructed into arguments when I first arrived at UT. My understanding has deepened due to the conversations that I had with Dr. Maynard.

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JoAnne drove me 215 miles from Rochester International Airport to Morristown, NY and then another 112 miles from Morristown to Syracuse International Airport.

Abstract

The Molecular Chaperone Skp Reduces Client Protein Aggregation Under *In Vivo-like* Conditions

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Recombinant proteins form the basis of a vibrant biotechnology industry, acting as key players in therapeutics, reagents and diagnostics.[1] However, multi-chain proteins are hard to produce efficiently.[1] The yield of recombinant proteins can be improved by co-expression of folding factors with the target protein.[2] Co-expression with folding factors has been met with some success.[2] However, deeper knowledge about the folding factors and the ways in which they interact with each other will ultimately improve expression of proteins.

The bacterial seventeen kilodalton protein (Skp) has been exploited to prevent aggregation of recombinant proteins in the bacterial periplasm.[3] While the interactions between Skp and its client proteins have been studied *in vitro*, the more relevant *in vivo* interactions have not, nor has its relationship to the other protein folding molecules in the bacterial periplasm. In particular, the order of interactions between Skp and the Disulfide bond isomerases A and C (DsbA, DsbC) is unclear. To address this knowledge gap, the effect of Skp on substrate protein aggregation was observed under *in vivo-like* conditions

as a step towards analyzing the effect of Skp *in vivo*. The effect of Skp on aggregation was reduced under *in vivo-like* conditions as compared to dilute *in vitro* experiments. The order of interaction between Skp, DsbA and DsbC on a substrate protein was observed. In the first experiment, the effect of the combination of Skp with DsbA and Skp with DsbC on the aggregation of lysozyme was observed. The greatest reduction in the aggregation of lysozyme was due to the addition of Skp with either DsbA or DsbC. In the second experiment, the order of interaction between Skp, DsbA and DsbC was assayed but no clear conclusion could be drawn. These insights will contribute to the understanding of protein expression in the bacterial periplasm.

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Chapter 1: Introduction

Recombinant proteins are used as reagents in laboratories and as therapeutics.[1] Recombinant proteins with disulfide bonds are expressed in the oxidizing periplasmic space as the environment in this space favors the formation of disulfide bonds.[1] Proteins destined for the periplasmic space are synthesized by ribosomes in the cytoplasm and are imported into the periplasmic space by the Sec translocon.[3] The folding of proteins is rate-limited by proline isomerization and by the formation of correctly paired disulfide bonds.[4] Proline isomerization is accelerated by peptidyl prolyl isomerases (PPIases), while the formation of correct disulfide bonds is facilitated by the disulfide bond (dsb) system.[3] Chaperones promote productive folding by sequestering unfolded proteins, thus allowing the protein more time to fold.[3] The yield of recombinant proteins can be improved by co-expression of folding factors with the target protein.[2] The use of periplasmic folding factors has been met with some success, but deeper knowledge about the folding factors individually and the ways in which they interact with each other will ultimately improve expression of proteins.[2] Basic research on protein folding will support research on protein folding diseases such as Alzheimers, Parkinson's and Cystic Fibrosis.

The bacterial Seventeen kilodalton protein (Skp) is a periplasmic holdase chaperone that prevents aggregation by sequestering unfolded proteins.[5] Skp has been exploited to prevent aggregation of recombinant proteins such as whole immunoglobulins (IgGs) and antibody fragments when expressed in the bacterial periplasm.[2] However, Entzminger *et al*. observed in 2012 that not all proteins interact with Skp and so Skp demonstrates client protein selectivity.[5] The basis for Skp client selectivity has not been fully elucidated. Also the order of interaction of Skp and the disulfide bond (dsb) formation system with substrate proteins has yet to be determined. The basis of Skp client

selectivity was further investigated in this work. The effect of Skp on substrate protein aggregation was observed under *in vivo-like* conditions as a step towards analyzing the effect of Skp *in vivo*. The order of interaction between Skp, DsbA and DsbC on a substrate protein was also observed. Further knowledge about Skp individually and interacting with other folding factors ultimately improve the production of soluble proteins.

VITAL OUTER MEMBRANE PROTEINS FOLD IN THE PERIPLASMIC SPACE

Recombinant proteins expressed in the periplasmic space of *Escherichia coli* take advantage of folding factors that help host proteins fold. The periplasmic space exists between the inner and outer membranes of E. coli.[3] Vital processes such as the transportation of nutrients, the rotation of flagella, and cell envelope repair occur in the periplasmic space.[3] As a result, 300 unique types of proteins can be found in this space.[3] These proteins fall into the broad classes of outer membrane proteins (OMPs), inner membrane proteins, soluble proteins and virulence factors. Outer membrane are an important class of proteins that perform a variety of functions.[6] For example, some OMPs, such as PhoE and LamB, allow the transport of nutrients into the cell.[6] Other OMPs, such as OmpT and phospholipase A, are enzymes.[6] The beta-barrel integral OMPs are especially well characterized.[7] The beta barrel of OMPs may be formed from 8-24 beta-strands and structure may contain a periplasmic domain.[7] The beta-barrel of OMPs spans the lipid bilayer.[7] Amino acids on the beta-strands that contact the hydrophobic tails of the lipid bilayer are hydrophobic.[7] Sections of the OMP protein sequence cross through the lipid bilayer are called transmembrane sequences (TMS).[7]

OMPs are inserted into the outer membrane by the BAM insertion complex.[3] OMPs are synthesized by ribosomes in the cytoplasmic space and cross the inner

membrane into the periplasmic space via the SecYEG translocon.[3] The SecYEG translocon is a channel embedded in the inner membrane.[3] Recombinant proteins that are destined for the periplasmic space contain a N-terminal signal sequence that is recognized by the SecYEG translocon.[3] The proteins are threaded linearly through SecYEG translocon and the signal sequence is cleaved upon arrival in the periplasmic space.[3] Hydrophobic residues in the peptide chain are exposed when the linearized protein exits the Sec translocon.[3] The hydrophobic residues on the unfolded peptide chain are attracted to the hydrophobic residues is other unfolded proteins thus causing aggregation.[3]

PERIPLASMIC FOLDING FACTORS AID IN SURVIVAL DURING HARSH CONDITIONS

Recombinant proteins expressed in the periplasmic space of *E. coli* take advantage of folding factors that help the cell survive in harsh environments.[8, 9] Cells respond to changes in environmental conditions through stress responses.[8, 9] Harsh environmental conditions such as high heat and variances in pH can denature proteins.[8, 9] The envelope stress response is elicited by the presence of unfolded proteins in the periplasmic space.[8, 9] Sigma factor σ^E (σ^{24}) upregulates the expression of periplasmic proteases, PPIases and molecular chaperones to assist in protein refolding and cleavage.[8, 9] Sigma factor σ^E is released by the detection of unfolded OMPs.[8, 9] Sigma factor σ^E is bound to RseA at the inner membrane on the cytoplasmic side under normal physiological conditions.[8, 9] RseA is an integral protein that contains a periplasmic and a cytoplasmic domain connected by one transmembrane region.[9] Under normal conditions RseB inhibits σ^E by binding to RseA.[9] OMP proteins contain a YxF sequence on the c-terminal of that is normally buried when an OMP is properly folded but it is exposed when the OMP protein has unfolded.[9] A protease called DegS binds to YxF sequences when they are exposed and becomes activated.[9] DegS and RseP trigger a proteolytic

cascade that cleaves RseA leaving one cytoplasmic domain of RseA with σ^E.[9] Protease ClpXP degrades the cytoplasmic domain of RseA releasing σ^E.[9] The binding of sigma factor σ^E to RNA polymerase drives the upregulation of folding factors such as FkpA and DegP.[8, 9] Conditions that denature proteins, such as heat or ethanol, induce this envelope stress response.[8, 9]

The two-component system of CpxA/CpxR responds to misfolded proteins, starvation, inner membrane alteration, and changes in pH.[8, 10] CpxA is a sensor histidine kinase and CpxR is a cytoplasmic response regulator CpxR.[8, 10] The kinase phosphorylates CpxR, which allows CpxR to bind to DNA and increase the expression of 150 proteins.[10] These proteins assist in the refolding of unfolded proteins and degradation of aggregates.[8, 10] These stress responses prevent the aggregation of proteins in the periplasmic space so that the cell can survive harsh conditions.

PERIPLASMIC FOLDING FACTORS PROMOTING FOLDING AND REDUCING AGGREGATION

The periplasmic space contains folding factors such as molecular chaperones, peptidyl prolyl isomerases (PPIases), disulfide bond isomerases that help the cell survive in harsh environments.[3] Protein aggregation in the periplasmic space is reduced through two redundant pathways.[3] The first pathway includes the chaperone and peptidyl-prolyl cis-trans isomerase (PPIase) SurA.[3] The second pathway includes the chaperone Skp and the protease DegP.[3] The two redundant pathways exist to ensure that proteins fold.[3] Mutation of *surA* results in hypersensitivity to EDTA, SDS, and bile salts.[3] This phenotype results because the outer membrane density is decreased due to decreased numbers of properly folded OMPs in *surA* mutant cells.[3] Mutation of *skp* or *degP* resulted in decreased amounts of LamB, OmpA, OmpC, OmpF.[3] Double mutants of *skp surA* and *skp surA* are lethal, indicating that at least one pathway is necessary for survival.[11] These

results established the notion that the two pathways were redundant.[3] Deletion of *surA* resulted in a more significant change in phenotype the deletion of *skp* or *degP*.[3] It was concluded from these results that SurA is the primary pathway for folding in the periplasmic space.[12] Skp and DegP comprise the secondary pathway, which rescues unfolded proteins that are not folded by the primary pathway.[12] The collective action of the chaperones in the periplasmic space is referred to as the chaperone network.[3] Substrate proteins may be able to interact with many different chaperones.[3] Substrate proteins that are dependent on a specific chaperone cannot fold productively without that specific chaperone.[3]

SurA, Skp, DegP, FkpA, DsbA, and DsbC are periplasmic folding factors that have exploited to improve the production of recombinant proteins.[2] A brief introduction to each factor is given here in order to explain how these factors improve recombinant protein folding. For a comprehensive review on periplasmic folding factors, read "Folding mechanisms of periplasmic proteins" by Camille Goemans *et al*.[3]

SurA is both a chaperone and a peptidyl-prolyl cis-trans isomerase (PPIase).[3, 13] The structure of SurA contains four domains.[3, 13] The domains include a N-terminal domain, two parvulin-like PPIase domains and a C-terminal domain.[3, 13] Only the second PPIase domain is functional.[3] The first PPIase, the N-terminal, and the C-terminal domains form the core of the protein where chaperone activity occurs.[3] The second PPIase is connected to the chaperone core by two long peptide chains.[3] SurA recognizes Ar-X-Ar motif in unfolded OMPs and is the primary chaperone for OMP folding.[3]

Seventeen kilodalton protein (Skp) is a periplasmic chaperone that prevents aggregation by sequestering unfolded proteins in its cage-like interior.[5] The native structure of Skp is homotrimeric and is comprised of three 17kDa monomers.[14] Each monomer contains two alpha helices and a beta sheet region.[14] The two alpha helices are

bent at an angle to form an arch.[14] The three monomers are loosely associated by a ring at the base formed from the beta sheet domains.[14] The final structure is a claw with three prongs.[14] Skp interacts with the linear periplasmic proteins immediately after they have crossed the inner membrane via the Sec translocon.[3] The unfolded substrate protein is inserted into the claw-like interior of Skp.[3]

DegP functions as a chaperone at low temperatures, but it can operate as a solely as a protease at high temperatures.[3, 8] DegP exists as a disordered, inactive homohexamer composed of two trimer rings.[3] Binding of unfolded substrate protein to PDZ domains and the active site initates the formation of a cage of 4-8 trimers.[3] This provides a large cavity to house unfolded proteins.[3] Binding of substrate activates the serine protease catalytic site for the degradation of proteins.[3]

FkpA is a chaperone and PPIase that assists protein folding in the periplasmic space.[3, 8] FkpA has been shown to assist Skp in the folding of LptD.[3] Double mutants of *skp* and *fkpA* results a loss of membrane integrity due to decreases in LptD.[3] *FkpA* is also upregulated by the envelope stress response.[8]

Correct disulfide bond formation is facilitated by the disulfide bond (dsb) system.[8, 15] Disulfide bonds are introduced into proteins by disulfide oxidoreductase A (DsbA).[8, 15] DsbA forms disulfide bonds by transferring two electrons stored in the disulfide bond in DsbA to target proteins.[8, 15] Reduced DsbA is reoxidized by DsbB.[8, 15] DsbA can only create disulfide bonds between consecutive cysteines.[8, 15] Some proteins contain non-consecutive disulfide bonds.[8, 15] Isomerization of incorrect disulfide bond pairing is catalyzed by DsbC. DsbC is regenerated by inner membrane protein DsbD.[8, 15]

CHAPERONES ENHANCE RECOMBINANT PROTEIN YIELD

This basic knowledge about periplasmic folding factors has been exploited to increase the yield of periplasmic proteins.[2] In 1998 Hendrick Bothmann and Andreas Pluckthun selected for periplasmic factors that could improve the folding of a single chain antibody fragment scFv) via phage display.[16] The structure of scFv includes the variable heavy (V_H) and variable light (V_L) domains connected by a flexible linker glycine serine linker $(Gly_4Ser)_3$.[17] Full-length antibodies are typically produced in mammalian cell lines.[17] ScFvs on average are one fifth of the size of full-length antibodies and so scFvs can be produced in *E. coli* with ease.[17] ScFvs retain antigen binding specificity but lack Fc effector constant domains.[17] The variable domains of the scFv can be engineered to improve aspects such as binding affinity or stability via phage display.[16] A library of phage displaying scFv variants is selected for over multiple generations.[16] The selected scFv variants contain advantageous mutations that improved their ability to surpass selection pressures.[16]

Bothmann and Pluckthun selected for periplasmic factors that could improve the folding of a scFv by digesting genomic *E. coli* DNA into 1-6 kb pieces and displaying the fragments on phage with poorly folding 4-4-20 scFv.[16] Selected phage were hypothesized to contain a fragment of *E. coli* DNA that led to the expression of a folding factor that may have assisted in the folding of the 4-4-20 scFv.[16] Skp was also shown to improve the folding of soluble 4-4-20 as shown by antigen binding ELISA.[16] The selected phage contained a 990 bp sequence insert.[16] This insert included the *E. coli skp* gene with a truncated sequence of the *lpxD* (*firA*) gene.[16] The ELISA result stayed the same when the *lpxD* gene was removed, indicating that the folding factor was due to *skp*, not *firA*.[16]

Skp has been exploited to improve the folding of many types of target proteins since then.[2] Expression of Skp was shown to improve the folding of scAb to pathogen

Brucella melitensis in phage display in 2003 by Andrew Hayhurst *et al*.[18] Skp has aided in the expression of single-chain alpha beta T-cell receptors.[19] In 2008 Lin *et al*. improved the yield of Fab59 100 fold through co-expression with Skp combined with codon optimization and adjustment to the promoter sequence.[20] Skp co-expresion also improves cell-surface by reducing extracytoplasmic stress.[21] Mitochondria-targeted Skp has even been used to improve the folding of the YadA autotransporter bacterial in yeast.[22]

Co-expression of Skp with additional of folding factors has become increasingly popular as our knowledge of the chaperone network has expanded.[2] In 2001 Georgiou *et al*. improved the expression of Fab antibody fragment in the cytoplasm of *E. coli* trxB gor mutants by adding Skp in addition to GroEL/S and TF and DsbC without a leader sequence.[1] In 2009 Georgiou improved the expression of human sialyltransferase ST6GalNAcl by co-expressing with Skp, TF, DnaK, DnaJ, and GroEL/ES. Co-expression with an additional folding factor can increase folding over folding due to Skp alone.[23]

Plasmids can now co-express six to seven folding partners with the target protein.[17] It was hypothesized by Sonoda *et al*. in 2011 that cocktails of multiple chaperones could be used to improve yield of recombinant proteins.[17] Anti-bovine ribonuclease A scFv 3A21 was co-expressed with sets of cytoplasmic and periplasmic chaperones.[17] Cytoplasmic chaperones co-expressed with the scFv included GroEL/ES, DnaK/DnaJ/GrpE, TF, and SecB.[17] Periplasmic chaperones co-expressed with the scFv included Skp and FkpA.[17] Chaperones did increase binding activity in all fractions as shown by ELISA and Western Blot.[17] The periplasmic chaperone set improved the binding activity of the scFv, but the cytoplasmic chaperone set did not have an effect.[17] Skp co-expression resulted in higher binding activity of the folded scFv than when FkpA was added.[17] These results demonstrated that generic cocktails of multiple chaperones are

not effective.[17] Further knowledge about the folding factors individually and in combination is needed in order to optimize protein production yields through the addition of folding factors.

While co-expression of Skp with target proteins dramatically increased the yield of the 4-4-20 scFv, it has been shown to be ineffective for some soluble proteins.[5] Entzminger *et al*. demonstrated in 2012 that soluble proteins exhibit varying sensitivities to Skp.[5] Three single chain variable fragments (scFvs) known to have varying sensitivies to Skp were observed.[5] The scFvs observed included ovalbumin peptide-MHC specific DO11.10 single-chain T cell receptor, 14B7 scFv specific to Protective Antigen and 4-4-20 scFv.[5]

Improvements in folding of the scFvs due to the addition of Skp was assessed as the scFvs were displayed on phage and expressed as soluble proteins.[5] Increases in folded scFv during phage display were monitored by phage ELISA.[5] The ELISA signal increased by significantly increased when Skp was co-expressed with 4-4-20 scFv.[5] A moderate increase was detected when Skp was co-expressed with 14B7, but a decrease was observed for DO11.10.[5] The impact of Skp on the folding of the scFvs expressed as soluble proteins was observed by western blot.[5] The soluble and insoluble fractions were compared for each scFv.[5] The level of scFv expressed in the soluble fraction over the insoluble fraction increased for 4-4-20 and 14B7 scFvs when Skp was co-expressed.[5] Skp co-expression did not alter the level of soluble protein expressed for DO11.10.[5] The DO11.10 scFv was determined to be Skp insensitive, the 14B7 scFv was determined to be somewhat Skp insensitive and 4-4-20 scFv was determined to be Skp dependent.[5] Thus, it was demonstrated that soluble proteins exhibit varying sensitivities to Skp.[5]

The knockdown of Skp was determined by Chen and Henning in 1996 to result in a decrease in the number of OMPs in the outer membrane.[24] Missiakas and Raina

observed an increase in DegP in *skp* knockdowns, which suggested to them that the σ^E response was induced.[24] This led Ute Schafer, Konstanze Beck and Matthias Muller to hypothesize in 1999 that proteins must be aggregated in the periplasmic space.[24] Troy Walton and Marcelo Sousa observed the impact of the addition of Skp on the aggregation of denatured lysozyme in 2004.[24] Lysozyme was denatured in urea and then rapidly diluted to a final concentration of 2μM.[24] Lysozyme was diluted into buffer or solutions with or without varying concentrations of Skp.[24] The absorbance was measured at 360nm, which is the wavelength of tryptophan emission.[5] Tryptophan residues are buried in folded proteins. An increase in tryptophan absorbance is expected to occur when proteins are unfolded.[5] Unfolded proteins aggregate, so an increase in absorbance at 360nm indicates an increase in aggregation.[5] Walton and Sousa observed that the addition of Skp decreased absorbance, which indicates a decrease in aggregation.[24]

Entzminger *et al*. observed the effect of Skp on the aggregation of soluble scFvs with varying Skp sensitivity using the aggregation assay developed by Walton and Sousa.[5] The impact of Skp on scFv folding was assayed by denaturing the scFvs over 6 hours in aggregation denaturation buffer (8M urea and 50mM DTT in HBS at pH 7.4) at a concentration of 100μM.[5] The scFvs were diluted 100 fold into HBS (10mM HEPES, 150 mM NaCl, pH 7.4) with and without maltose binding protein (MBP) or Skp at 1, 3, and 6μM.[5] Aggregation was observed at 360nm over 30 minutes.[5] The aggregation of Skp-sensitive scFvs decreased as the concentration of Skp increased.[5] Addition of Skp to Skp-insensitive proteins did not reduce the aggregation of scFvs.[5] The impact of Skp on scFv folding was also observed.[5] However, it was determined that Skp did not have an impact on folding.[5] This supported the conclusion that Skp acts as a holdase chaperone and not a foldase chaperone.[5]

The mechanism of interaction between Skp and substrate proteins has not been determined. Entzminger *et al*. postulated that Skp preferentially interacts with proteins containing a folding intermediate.[5] Folding intermediates of the scFvs were probed through stability tests.[5] First, stability in denaturant was assayed.[5] The scFvs were denatured in varying concentrations of urea.[5] Tryptophan fluorescence was observed. Unfolding occurs at 323nm for DO11.10 and 360nm for 14B7.[5] The excitation wavelength was 280nm, and emission wavelengths of 310-380nm were recorded.[5] DO11.10 was determined not to have an intermediate, but 14B7 scFv did have one intermediate.[5] The 4-4-20 scFv could not be analyzed as it was poorly soluble. Secondly, thermal stability was determined by incubating 10μM of scFvs with 1μL of 1:1000 Sypro Orange at temperatures ranging from 20-90°C.[5] No conclusive result was derived through thermal stability testing.[5] Thus it was demonstrated that Skp sensitive proteins contained a stable folding intermediate *in vitro*.[5]

RESEARCH GOALS

The basis of Skp client selectivity was further investigated in this work. The effect of Skp on substrate protein aggregation was observed under *in vivo-like* conditions as a step towards analyzing the effect of Skp *in vivo*. The individual role of Skp will be explored in Chapter 2. Also, the order of interaction of Skp and the disulfide bond (dsb) formation system with substrate proteins has yet to be determined. The order of interaction between Skp, DsbA and DsbC on a substrate protein was observed. The role of Skp with other periplasmic folding factors will be explored in Chapter 3. Further knowledge about the role of Skp individually and with other periplasmic folding factors will ultimately improve the production of soluble proteins.

Chapter 2: Macromolecular Crowding

INTRODUCTION

Skp improves the yield of proteins *in vivo*.[2] Entzminger *et al*. demonstrated that Skp decreases the aggregation of Skp-sensitive proteins *in vitro*.[5] The aggregation assay utilized was conducted under dilute conditions.[25] Dilute *in vitro* experiments typically contain 1-10 gl^{-1} of macromolecules.[25] In reality, cell interiors are crowded with macromolecules.[25] The cell interior is estimated to be up to 30% crowded by macromolecules (300 gl^{-1}).[25] A concentrated solution refers to a solution with a large number of molecules of a single species in a specified volume, whereas a crowded solution is a solution containing different macromolecules present at large numbers in a specified volume.[25] Crowding refers to the to the volume excluded by macromolecules.[25] Thermodynamic equilibrium measurements and reaction rates *in vivo* can differ by orders of magnitude from values obtained *in vitro*.[25] Protein folding alters the available volume so it is apropos to observe Skp under *in vivo-like* conditions that approximate the degree of crowding in the cell. The effect of Skp on substrate protein aggregation was observed under *in vivo-like* conditions as a step towards elucidating the basis for Skp client selectivity *in vivo*.

In vivo-like conditions can be achieved by adding high concentrations of a crowding agent to *in vitro* experiments.[25] Successful crowding agents are globular, transparent and inert while have low viscosity. Popular crowding agents include Ficoll, Dextran, PEG, and sucrose.[25] Ficoll 70 (Sigma) was chosen for this investigation as it fulfills the criteria of a successful crowding agent and also behaves as a rigid sphere.[25]

The effect of Skp on the aggregation of lysozyme in a crowded *in vivo-like* environment was observed. Proteins with a high degree of purity were generated for use in subsequent experiments. Next, the lysozyme aggregation assay from Entzminger *et al*.

was replicated as a control experiment.[5] Then the lysozyme aggregation assay was performed under *in vivo-like* conditions with varying percentages of Ficoll 70. Lastly, the lysozyme aggregation assay was performed under *in vivo-like* conditions with varying percentages of Ficoll 70 with the addition of Skp. Knowledge about the effect of Skp on aggregation under *in-vivo like* conditions will be the first step towards elucidating the basis for Skp client selectivity *in vivo*.

MATERIALS AND METHODS

Protein Purification

Soluble Skp protein was expressed in the cytoplasmic space of *E. coli* as outlined in Entzminger *et al*.[5] The *skp* gene without a leader sequence was previously cloned into a pET100D-TopoTA (Invitrogen) plasmid containing an n-terminal hexahistidine tag to facilitate protein expression in BL21(DE3) cells.[5] Cells were grown in Terrific Broth with 200 μg/mL ampicillin by shaking at 225rpm at 37°C until an OD_{600} of 0.6 was reached. Expression of Skp was induced by adding 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were grown for 5 hours with shaking at 225rpm at 25°C. The cells were pelleted and resuspended in French Press Buffer (25 mM HBS pH 7.4, 500 mM NaCl, 40 mM Imidazole). Cells were lysed by passing the cells through the French Press (Thermo FA-078A) at 1500 psi pressure in a 35mL French Press cell (Thermo FA-032). Cellular debris was pelleted and the supernatant containing Skp protein was dialyzed overnight. The protein was eluted by Ni^{2+} immobilized metal affinity chromatography (IMAC). The eluted protein was purified in a second step through Superdex S75 or S200 size exclusion chromatography (SEC) columns operating on the AKTA FPLC system (GE Healthcare). The running buffer was HBS (pH 7.4). The SEC columns were standardized with proteins with a broad range of molecular weights (GE Healthcare, Sigma). The molecular

weight of eluted proteins can be determined relative to the molecular weight standards. The molecular weight of the eluted protein was confirmed by 12% SDS-PAGE. The concentrations of protein samples were determined by BCA Assay (Pierce). Lyophilized Bovine Serum Albumin (BSA, 2mg/mL) was used as a standard.

Soluble Maltose Binding Protein (MBP) was expressed in the periplasmic space of *E. coli* as outlined in Entzminger *et al*.[5] The MBP gene was previously cloned into a pAK400 plasmid with a n-terminal hexahistidine tag in BL21 (DE3) cells. Cells were grown in a starter culture of 2mL TB with 34μg/mL Cloramphenicol with shaking at 225rpm at 37°C until an OD_{600} 0.5 was exactly reached. Cells were grown in Terrific Broth with 200 μg/mL ampicillin by shaking at 225rpm at 37°C until an OD_{600} of 0.6 was reached. This starter culture was used to inoculate 250mL TB containing 34μg/mL Cloramphenicol. Cells were grown overnight with shaking at 225rpm at 37°C. The media was exchanged for 250mL of fresh TB with 34μg/mL Chloramphenicol. The cells were allowed to recover for 1 hour at 25°C and then expression of MBP was induced by adding a final concentration of 1mM IPTG. The cells were grown for 5 hours with shaking at 225rpm at 25°C. The cells were harvested and lysed by osmotic shock. Cells were resuspended in 10 mL sucrose solution (0.75M sucrose, 0.1M Tris, pH 8.0) and transferred into four 50mL centrifuge tubes. Additionally 10mL sucrose solution was added to each tube along with 250mL of 10mg/mL lysozyme in sucrose solution and 10mL of 1mM EDTA (pH 8.0). The cells were incubated with rotation at 4C for 20-60 minutes. Then, 0.5mL Magnesium Chloride ($MgCl_2$) was added to each tube. The cells were incubated with rotation at 4C for 10 minutes. Cellular debris was pelleted and the supernatant containing MBP protein was dialyzed overnight. The protein was eluted by Ni^{2+} immobilized metal affinity chromatography (IMAC). The eluted protein was purified in a second step through Superdex S75 or S200 size exclusion chromatography (SEC)

columns operating on the AKTA FPLC system (GE Healthcare). The identify of the purified protein was characterized by 12% SDS-PAGE and the quantity of protein produced was determined by BCA Assay.

Aggregation Assay

The aggregation of lysozyme was replicated as proscribed by Entzminger *et al*.[5] Lysozyme was equilibrated in denaturation buffer (8M urea, 50mM, pH 7.4) for 6 hours at a concentration of 100μM. Denatured lysozyme was rapidly diluted 100 fold into HBS (10mM HEPES, 150mM NaCl, pH 7.4) solutions alone or with MBP or Skp in varying concentrations of Ficoll 70 crowding agent (0-30%). Lysozyme in denaturation buffer was added to the wells of a black, clear bottom 96-well Costar plate. The HBS solution with or without MBP or Skp in varying concentrations of Ficoll 70 crowding agent was pipetted into the denatured lysozyme solution. The absorbance at 360nm was detected at 10s intervals over 30 minutes at room temperature with a SpectraMax M5. Experiments were replicated in triplicate. The background absorbance was subtracted from the average of the three replicates. The curves were normalized by dividing by the largest difference in absorbance. The error bars indicate the standard deviation of three replicates.

RESULTS

The proteins used in subsequent experiments in this study had a high degree of purity as determined by size exclusion chromatography (SEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Skp was expressed in the cytoplasmic space, purified first by IMAC chromatography and was purified next by SEC. The expected molecular weight of native trimeric Skp with a his-tag is estimated from the sequence to be 70,440 Da. A peak in the absorbance at 280nm is expected to occur between the Conalbumin (75kDa, 9.015mL) and Albumin (66kDa, 9.051mL)

standard elution volumes. A peak was detected in this elution volume range as shown in Figure 2.1, confirming the identity of the produced protein. Skp does not contain any tryptophan or cysteine residues, so the absorbance at 280nm is low even a total of 1.42mg of trimeric Skp was purified.

The purity of the protein was also confirmed by gel electrophoresis on a 12% SDS-Polyacrylamide gel as shown in Figure 2.2. A ladder (NEB Pre-stained Protein Marker, Broad Range) is shown in lane 4. Skp is expected to be monomeric under reducing conditions. The molecular weight of monomeric Skp containing a his tag is expected to be 25kDa. The protein samples in lanes 2,3,5-15 migrated as far as the 25kDa marker. This further confirmed that Skp was produced. A single band is observed in lane of protein samples indicating that the protein was pure. A BCA Assay confirmed that sufficient quantities of protein were produced in order to proceed.

MBP was expressed in the periplasmic space, purified first by IMAC chromatography and was purified next by SEC. MBP is natively found as a monomer. The expected molecular weight of MBP containing a his tag is estimated from the sequence to be 44,500 Da. A peak in the absorbance at 280nm is expected to occur between the Albumin (66kDa, 9.051mL) and Ovalbumin (44kDa, 9.633mL) standard elution volumes. A peak was detected in this elution volume range as shown in Figure 2.3, confirming the identity of the produced protein.

The purity of the protein was also confirmed by gel electrophoresis on a 12% SDS-Polyacrylamide gel as shown in Figure 2.4. A ladder (NEB Pre-stained Protein Marker, Broad Range) is shown in lane 3. The molecular weight of monomeric MBP with a his tag is expected to be 46,000 Da. The protein samples in lanes 4-15 migrated as far as the 46 kDa marker. This further confirmed that MBP was produced. A single band

is observed in lane of protein samples indicating that the protein was pure. A BCA Assay confirmed that sufficient quantities of protein were produced in order to proceed.

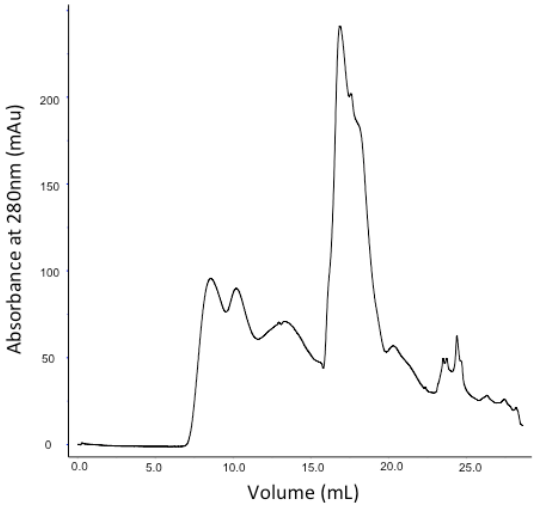
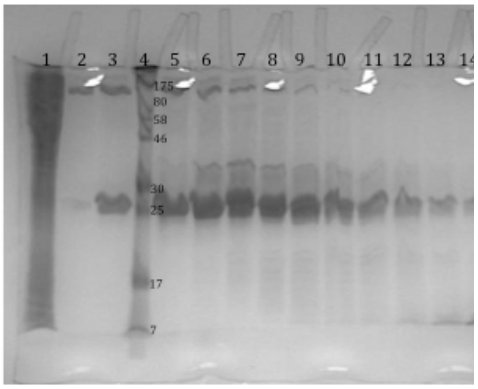


Figure 2.1: Skp was produced as shown by size exclusion chromatography on a GE Healthcare Superdex S75 column on the AKTA FPLC system. The molecular weight of trimeric Skp is 70kDa. Trimeric Skp eluted between 9.015-9.510mL. The third peak from the left corresponds to a total of 1.42mg of trimeric Skp.



Lanes

1. SKP_ Flow through
2. SKP_A6
3. SKP_A7
4. Ladder
5. SKP_A8
6. SKP_A9
7. SKP_A10
8. SKP_A11
9. SKP_A12

Lanes

10. SKP_B12
11. SKP_B11
12. SKP_B10
13. SKP_B9
14. SKP_B8
15. SKP_B7

Figure 2.2: Recombinant Skp preparations exhibited a high degree of purity. Different fractions from a purification run are shown, separated on a 12% SDS-Poly Acrylamide Gel. Lanes reflect different fractions collected including the flowthrough from the IMAC column and those eluted off an S75 size exclusion chromatography column on an Akta FPLC. The molecular weight of monomeric Skp with a his tag is expected to be 25kDa. The protein samples in lanes 2,3,5-15 migrated as far as the 25kDa marker. This further confirmed that Skp was produced. A single band is observed indicating that the preparations were pure.

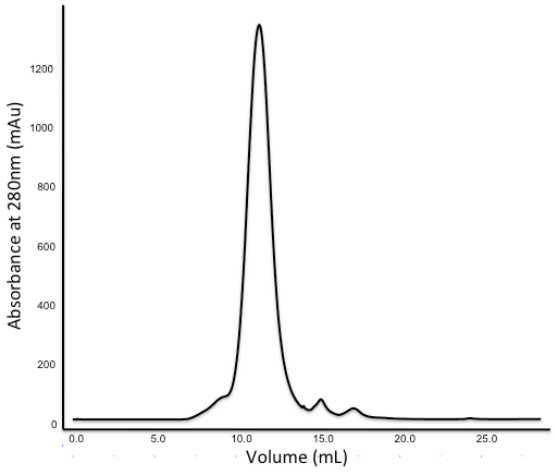
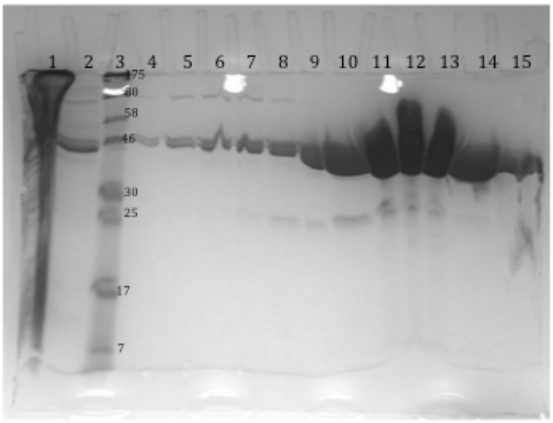


Figure 2.3: MBP was produced as shown by size exclusion chromatography on a GE Healthcare Superdex S75 column on the AKTA FPLC system. The molecular weight of monomeric MBP is 44kDa. Monomeric MBP eluted between 9.633-11.122mL. The peak from the left corresponds to a total of 5.83mg of MBP.



Lanes		Lanes
1.	Ladder	10. MBP_A12
2.	MBP_Lysate	11. MBP_B12
3.	MBP_Flow through	12. MBP_B11
4.	MBP_A6	13. MBP_B10
5.	MBP_A7	14. MBP_B9
6.	MBP_A8	15. MBP_B8
7.	MBP_A9	
8.	MBP_A10	
9.	MBP_A11	

Figure 2.4: Recombinant MBP preparations exhibited a high degree of purity. Different fractions from a purification run are shown, separated on a 12% SDS-Poly Acrylamide Gel. Lanes reflect different fractions collected including the flowthrough from the IMAC column and those eluted off an S75 size exclusion chromatography column on an Akta FPLC. The molecular weight of monomeric MBP with a hist tag is 46kDa. The protein samples migrated as far as the 46kDa marker. This further confirmed that MBP was produced. A single band is observed indicating that the preparations were pure.

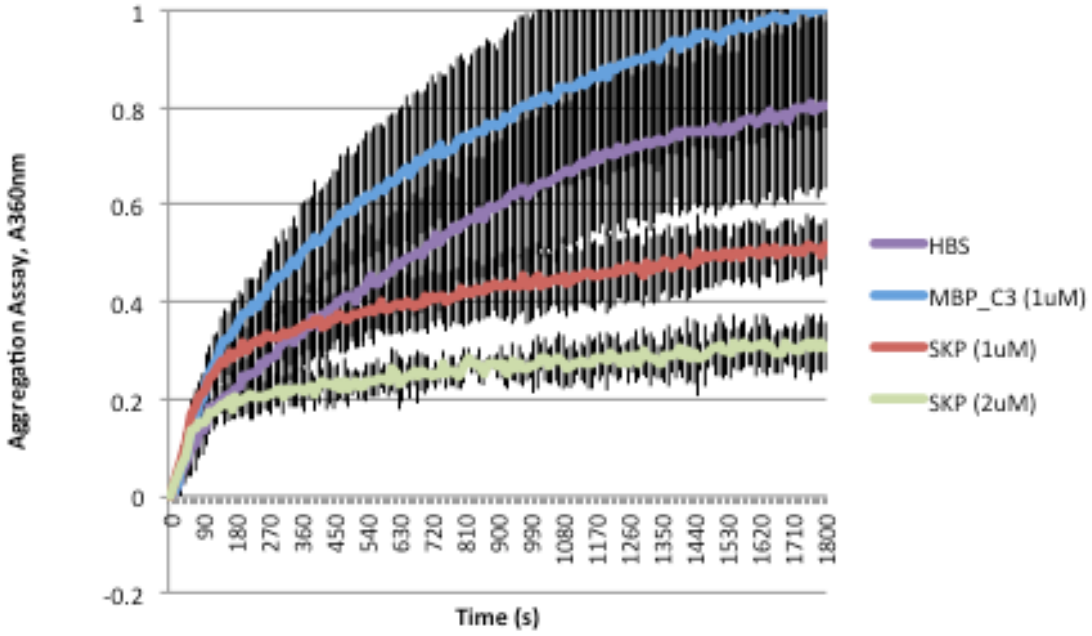


Figure 2.5: Skp reduces the aggregation of proteins *in vitro* as demonstrated in a lysozyme aggregation assay. Denatured lysozyme was added to solutions containing HBS, MBP (1μM) or Skp (1 or 2μM). MBP does not exhibit chaperone activity, so it was used as a negative control. Absorbance does not decrease when MBP was added indicating that MBP does not reduce the aggregation of proteins. Absorbance was reduced proportionally with the addition of Skp indicating that Skp does reduce aggregation. The error bars represent the standard deviation of three replicates.

The Lysozyme Aggregation Assay from Entzminger *et al*. was replicated as a control experiment.[5] Lysozyme was rapidly diluted into HBS alone or with 1μM MBP or Skp (1-2μM) as shown in Figure 2.5. Lysozyme contains hydrophobic residues that are exposed when the protein is denatured.[5] Unfolded lysozyme proteins are expected to aggregate.[5] Absorbance at 360nm increased over time when lysozyme was rapidly diluted into HBS solution. The aggregation of lysozyme increased over time in HBS. MBP does not exhibit chaperone activity, and so it was used a negative control. Absorbance at 360nm increased over time when lysozyme was rapidly diluted into HBS with 1μM

MBP. The aggregation of lysozyme also increased over time in HBS with 1μM MBP as expected. Skp is expected to reduce aggregation. A decrease absorbance at 360nm over time indicates that Skp reduced aggregation. The absorbance at 360nm decreased over time when Skp was added to denatured lysozyme. The absorbance decreased proportionally to the amount of Skp added as was shown by Entzminger *et al*.[5] Aggregation results in a heterogeneous population of monomers, dimers and trimmers. The standard error increased over time as the reaction progressed because the population of aggregates became more diverse.

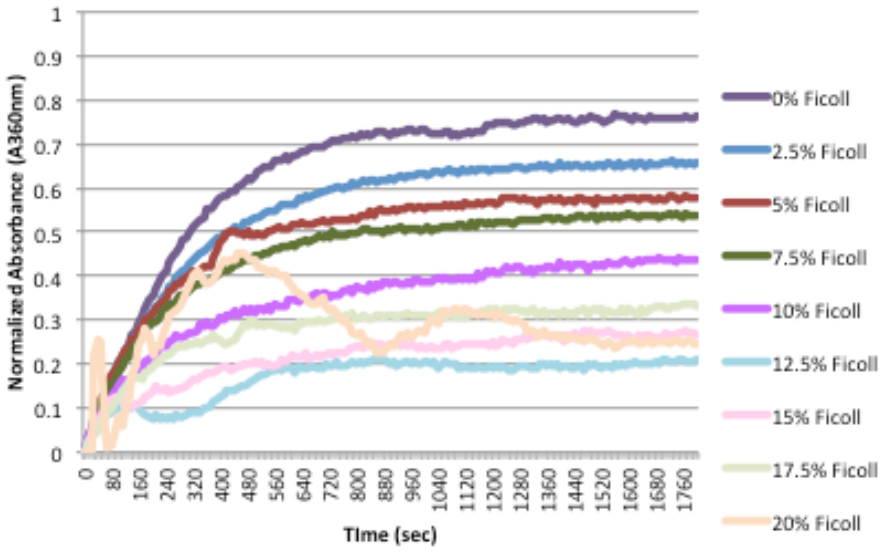


Figure 2.6: Low levels of crowding reduce the aggregation of proteins, but high levels of crowding increase aggregation as shown in a lysozyme aggregation assay. Denatured lysozyme was added to solutions containing varying percentages of Ficoll 70 crowding agent. Low levels of crowding agent are expected to promote the folding of proteins and decrease aggregation, whereas high levels of crowding agent are expected to cause aggregation. The amount of absorbance, thus aggregation, over time decreased as concentrations of Ficoll increased up to 12.5%. The absorbance increased as concentrations of Ficoll increased from 12.5% to 20%. Low concentrations of Ficoll reduced aggregation as predicted, while high concentrations increased aggregation.

The lysozyme aggregation assay was performed under *in vivo-like* conditions with varying percentages of Ficoll 70 but without Skp. A small amount of crowding promotes the folding of the substrate because the decrease in entropy is favored. Low concentrations of Ficoll are expected to increase the amount of folded protein and thus decrease the amount of aggregation. High concentrations of Ficoll are expected to cause aggregation. The amount of absorbance, thus aggregation, over time decreased as concentrations of Ficoll increased up to 12.5%. The absorbance increased as

concentrations of Ficoll increased from 12.5% to 20%. Low concentrations of Ficoll reduced aggregation as predicted, while high concentrations increased aggregation. These results confirmed the initial prediction.

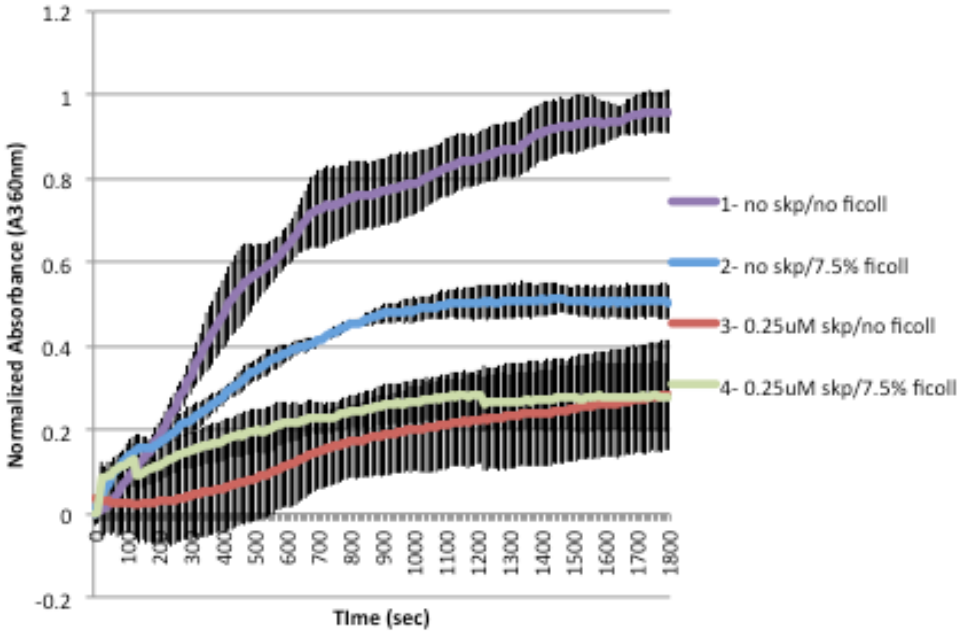


Figure 2.7: The effect of Skp on aggregation is diminished under *in vivo-like* conditions as compared to dilute *in vitro* experiments. Denatured lysozyme was diluted in solutions containing HBS or 7.5% ficoll with and without Skp (1μM). The greatest reduction in absorbance, and thus aggregation, resulted when Skp was added under dilute conditions. Skp reduced aggregation under crowded conditions. However, a reduction in aggregation was observed under *in vivo-like* conditions as compared to dilute *in vitro* experiments.

The lysozyme aggregation assay was performed under *in vivo-like* conditions with varying percentages of Ficoll 70 and varying concentrations of Skp. Skp was shown to decrease aggregation in Figure 2.5. Also, low levels of Ficoll 70 were shown to decrease aggregation in Figure 2.6. The addition of Skp in the presence of low levels of Ficoll is expected to decrease aggregation. The addition of 0.25μM Skp in the presence of 7.5% Ficoll was observed to have higher aggregation than Skp without crowder as shown in

Figure 2.7. The prediction that Skp and low concentrations of Ficoll would decrease aggregation was incorrect. The effect of Skp on aggregation under *in vivo-like* conditions may be diminished as compared to *in vitro* results as the diffusion of Skp may be impeded in a crowded environment.

CONCLUSIONS

The effect of Skp on the aggregation of lysozyme in a crowded *in vivo-like* environment was observed. The lysozyme aggregation assay was performed under *in vivo-like* conditions with varying percentages of Ficoll 70. It was demonstrated that low concentrations of Ficoll reduced aggregation while high concentrations increased aggregation. The lysozyme aggregation assay was performed under *in vivo-like* conditions with varying percentages of Ficoll 70 with the addition of Skp. The effect of Skp on aggregation under *in vivo-like* conditions was diminished as compared to the effect of Skp in dilute *in vitro* conditions. This result may have occurred as the diffusion of Skp is decreased in a crowded environment. Further experiments observing the effect of Skp in varying concentrations of crowding agents would provide a sharper picture of Skp under *in-vivo like* conditions.

Chapter 3: Disulfide Bond Formation in the Periplasmic Space

INTRODUCTION

The order of interaction of Skp and the disulfide bond (dsb) formation system with substrate proteins has yet to be determined. Silhavy *et al*. investigated the order of interaction SurA, dsbA, and dsbC with lilpoprotein (LptD) in 2010.[26] LptD is an outer integral membrane protein that is involved with the transfer of lipopolysaccharide to the outer leaflet of the outer membrane.[26] LptD preferentially interacts preferentially with SurA and forms a complex with LptE.[26] LptD contains two non-consecutive disulfide bonds.[26] LptD is stable and can fold without disulfide bond formation.[26] However, LptD cannot function unless one non-consecutive disulfide bond is correctly formed. These results suggest that LptD interact with SurA before the dsb system.[26] A similar investigation into the order of interaction between Skp, DsbA and DsbC with substrate proteins has not occurred. Insights into the order of interaction of a second chaperone with DsbA and DsbC will comment on the larger discussion on protein folding. This knowledge will ultimately improve the production of soluble proteins.

The order of interaction between Skp, DsbA and DsbC on a substrate protein was investigated. First, a lysozyme aggregation assay was performed with varying combinations of Skp, DsbA and DsbC. Skp and DsbA or DsbC is predicted to have the greatest effect in decreasing aggregation. The second experiment determined the order of interaction of Skp, DsbA, and DsbC with substrate protein. A lysozyme aggregation assay was performed under two conditions. In the first condition, DsbC was incubated with denatured lysozyme for 2 minutes, and then Skp was added. The absorbance at 360nm was monitored over 30 minutes. In the second condition, Skp was incubated with lysozyme for two minutes, and then DsbC was added. The absorbance was again monitored at 360nm for 30 minutes. Determining the order of disulfide bond interaction

will inform the larger conversation on protein folding. Further experiments are needed to understand this interaction.

MATERIALS AND METHODS

Protein Purification

Soluble DsbA and DsbC were expressed in the periplasmic space of *E. coli* using the same purification scheme as MBP in Chapter 2. The DsbA gene was previously cloned into a pAK400 plasmid with a n-terminal hexahistidine tag in BL21 (DE3) cells. DsbC was similarly cloned into a separate pAK400 plasmid in BL21 (DE3) cells. The eluted protein was purified in a second step through Superdex S75 or S200 size exclusion chromatography (SEC) columns operating on the AKTA FPLC system (GE Healthcare). The identify of the purified protein was characterized by 12% SDS-PAGE and the quantity of protein produced was determined by BCA Assay.

Aggregation Assay

The aggregation of lysozyme was replicated as in Chapter 2. In the first experiment in Figure 3.5 five HBS were assayed: HBS only, DsbC, DsbC with Skp, DsbA with Skp and DsbA. The absorbance at 360nm was detected at 10s intervals over 30 minutes at room temperature with a SpectraMax M5.

In the second experiment the aggregation assay was modified. This assay assessed the order of interaction between DsbC and Skp. Two experiments were tested. First, DsbC was added to denatured lysozyme and allowed to incubate for 2 minutes. Then, Skp was added. The absorbance at 360nm was detected at 10s intervals over 30 minutes at room temperature with a SpectraMax M5 immediately after the addition of Skp. Secondly, Skp was added to denatured lysozyme and allowed to incubate for 2 minutes.

Then, DsbC was added. The absorbance at 360nm was detected at 10s intervals over 30 minutes at room temperature immediately after the addition of DsbC.

RESULTS

The proteins used in subsequent experiments in this study had a high degree of purity as determined by size exclusion chromatography (SEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). DsbA was expressed in the periplasmic space, purified first by IMAC chromatography and was purified next by SEC. DsbA exists as a monomer natively. The expected molecular weight of monomeric DsbA with a his-tag is estimated from the sequence to be 21,100 Da. A peak in the absorbance at 280nm is expected to occur between the Carbonic Anhyrdrase (29kDa, 11.122mL) and Cytochrome C (12.4 kDa, 12.792mL) standard elution volumes. A peak was detected in this elution volume range as shown in Figure 3.1, confirming the identity of the produced protein.

The purity of the protein was also confirmed by gel electrophoresis on a 12% SDS-Polyacrylamide gel as shown in Figure 3.2. A ladder (NEB Pre-stained Protein Marker, Broad Range) is shown in lane 4. The molecular weight of monomeric DsbA with a his tag is expected to be 23kDa. The protein samples in lanes 2,3 and 4-15 migrated between the 17 and 25kDa markers. This further confirmed that DsbA was produced. A single band is observed in lane of protein samples indicating that the protein was pure. A BCA Assay confirmed that sufficient quantities of protein were produced in order to proceed.

DsbC was expressed in the periplasmic space, purified first by IMAC chromatography and was purified next by SEC. DsbC is natively found as a dimer. The expected molecular weight of DsbC is estimated from the sequence to be 47,000 Da. A

peak in the absorbance at 280nm is expected to occur between the Albumin (66kDa, 9.051mL) and Ovalbumin (44kDa, 9.633mL) standard elution volumes. A peak was detected in this elution volume range as shown in Figure 3.3, confirming the identity of the produced protein.

The purity of the protein was also confirmed by gel electrophoresis on a 12% SDS-Polyacrylamide gel as shown in Figure 3.4. A ladder (NEB Pre-stained Protein Marker, Broad Range) is shown in lane 5. DsbC is a monomer under reducing conditions. The molecular weight of monomeric DsbC with his tag is expected to be 25kDa. The protein samples in lanes 2-4 and 6-15 migrated as far as the 25kDa marker. This further confirmed that DsbC was produced. A single band is observed in lane of protein samples indicating that the protein was pure. A BCA Assay confirmed that sufficient quantities of protein were produced in order to proceed.

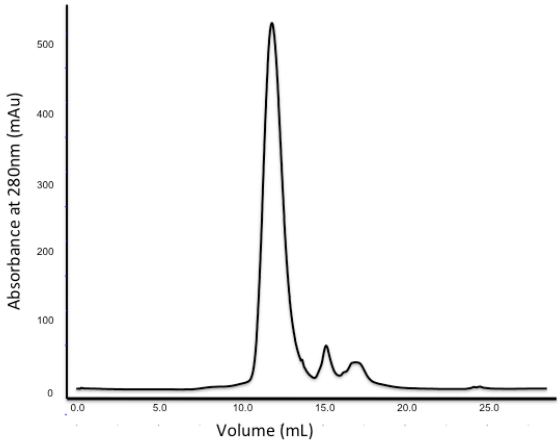
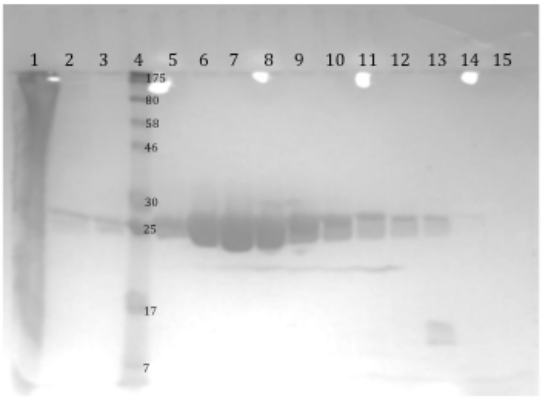


Figure 3.1: DsbA was produced as shown by size exclusion chromatography on a GE Healthcare Superdex S75 column on the AKTA FPLC system. The molecular weight of monomeric DsbA is 21kDa. Monomeric DsbA eluted between 11.122-12.792mL. The peak corresponds to a total of 2.03mg of monomeric DsbA.



Lanes:

1. dsbA_Lysate
2. dsbA_Flow through
3. dsbA_A12
4. Ladder
5. dsbA_B12
6. dsbA_B11
7. dsbA_B10
8. dsbA_B9
9. dsbA_B8
10. dsbA_B7
11. dsbA_B6
12. dsbA_B5
13. dsbA_B4
14. dsbA_B1
15. dsbA_C1

Figure 3.2: Recombinant DsbA preparations exhibited a high degree of purity. Different fractions from a purification run are shown, separated on a 12% SDS-Poly Acrylamide Gel. Lanes reflect different fractions collected including the flowthrough from the IMAC column and those eluted off an S75 size exclusion chromatography column on an Akta FPLC. The molecular weight of monomeric DsbA with a his tag is expected to be 23kDa. The protein samples migrated as far as the 25kDa marker. This further confirmed that DsbA was produced. A single band is observed indicating that the preparations were pure.

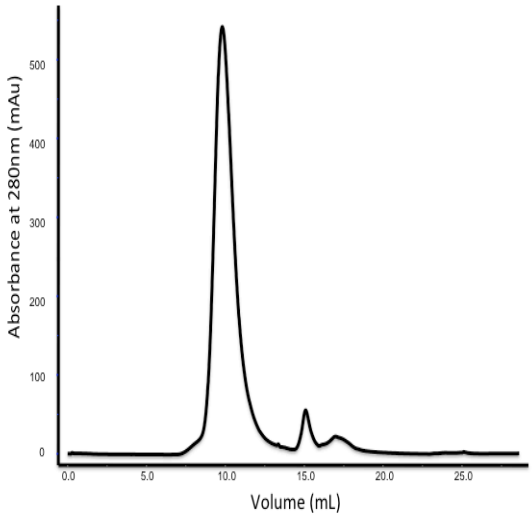
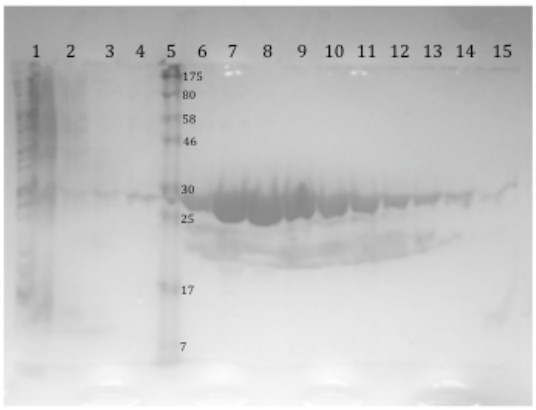


Figure 3.3: DsbC was produced as shown by size exclusion chromatography on a GE Healthcare Superdex S75 column on the AKTA FPLC system. The molecular weight of dimeric DsbC is 47kDa. Dimeric DsbC eluted between 9.051-9.633mL. The peak corresponds to a total of 3.57mg of dimeric DsbC.



Lanes:

1. dsbC_Lysate
2. dsbC_Flow through
3. dsbC_A7
4. dsbC_A8
5. Ladder
6. dsbC_A9
7. dsbC_A10
8. dsbC_A11
9. dsbC_A12

Lanes:

10. dsbC_B12
11. dsbC_B11
12. dsbC_B10
13. dsbC_B9
14. dsbC_B8
15. dsbC_B4

Figure 3.4: Recombinant DsbC preparations exhibited a high degree of purity. Different fractions from a purification run are shown, separated on a 12% SDS-Poly Acrylamide Gel. Lanes reflect different fractions collected including the flowthrough from the IMAC column and those eluted off an S75 size exclusion chromatography column on an Akta FPLC. The molecular weight of monomeric DsbC with a his tag is 25kDA. The protein samples in lanes 2-4, and 6-15 migrated as far as the 25kDa marker. This further confirmed that DsbC was produced. A single band is observed indicating that the preparations were pure.

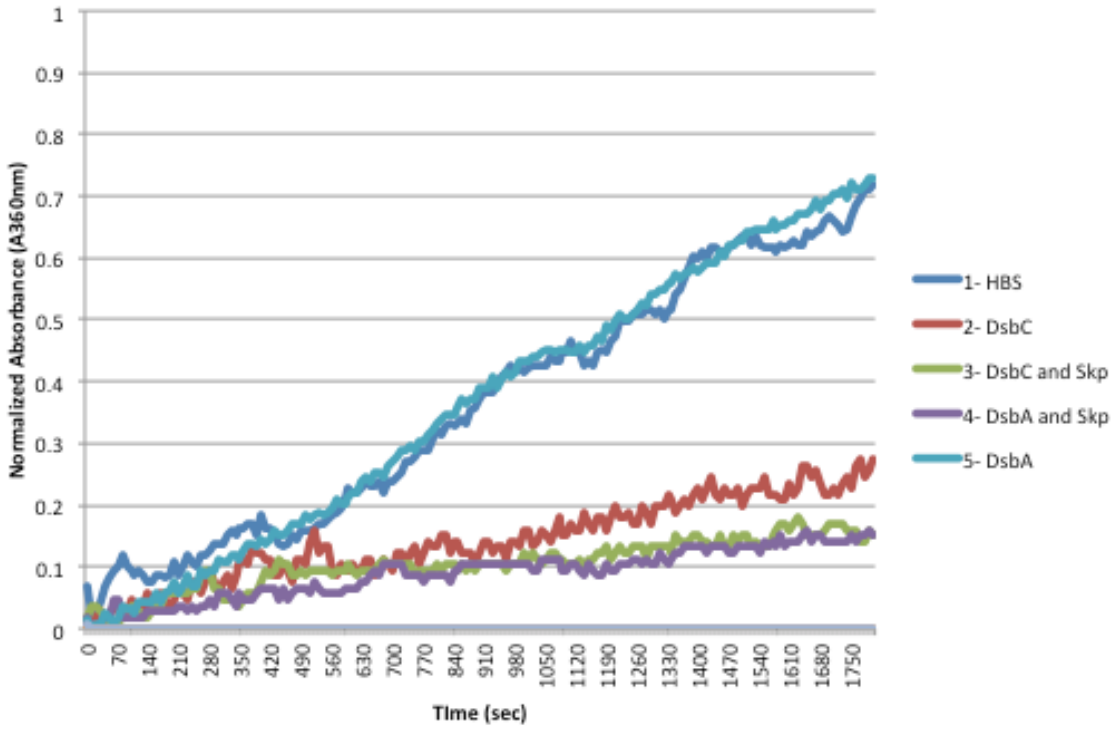


Figure 3.5 Aggregation was reduced the most by the addition of DsbC as shown in this lysozyme aggregation assay. Denatured lysozyme was diluted in solutions containing HBS, DsbC, DsbC with Skp, DsbA with Skp and DsbA. The error bars show the standard deviation of three replicates.

First, the impact of Skp interacting with DsbA and DsbC was probed as shown in Figure 3.5. Denatured lysozyme was rapidly diluted into five different HBS solutions. The five conditions were HBS alone, DsbC, DsbC with Skp, DsbA with Skp and DsbA. The addition of Skp with either DsbA or DsbC is expected to reduce aggregation the most. The greatest reduction in the aggregation of lysozyme was due to the addition of Skp with either DsbA or DsbC. DsbC greatly reduced the absorbance, but not to the same level as Skp with either DsbA or DsbC. The isomerization of incorrectly paired disulfide

bonds reduces aggregation significantly. No decrease in absorbance was observed with the addition of DsbA. Protein aggregation is reduced primarily through Skp and DsbC.

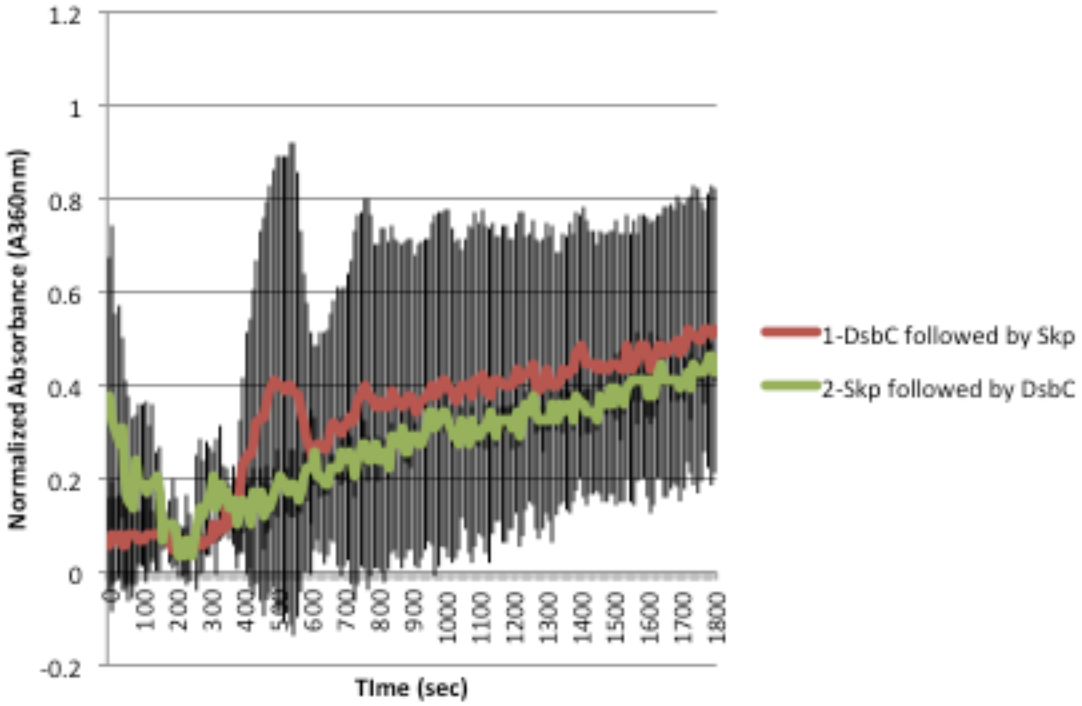


Figure 3.6 The order of interaction between DsbC and Skp as shown in a lysozyme aggregation assay. In the first reaction, DsbC was incubated with denatured lysozyme for two minutes then Skp was added and the absorbance was observed at 360nm for 30 minutes. In the second reaction, Skp was incubated with denatured lysozyme for two minutes then DsbC was added and the absorbance was observed at 360nm for 30 minutes. The trends are not statistically significant and further experimentation is needed to distinguish the order of interaction between Skp and DsbC.

The order of interaction of Skp with DsbC was observed in Figure 3.6. In the first reaction, DsbC was incubated with denatured lysozyme for two minutes then Skp was added and the absorbance was observed at 360nm for 30 minutes. In the second reaction, Skp was incubated with denatured lysozyme for two minutes then DsbC was added and the absorbance was observed at 360nm for 30 minutes. The trends are not statistically

significant and further experimentation is needed to distinguish the order of interaction between Skp and DsbC.

CONCLUSIONS

The order of interaction of Skp, DsbA and DsbC with a substrate protein was investigated. In the first experiment, the effect of the combination of Skp with DsbA and Skp with DsbC on the aggregation of lysozyme was observed. The greatest reduction in the aggregation of lysozyme was due to the addition of Skp with either DsbA or DsbC. In the second experiment, the order of interaction between Skp, DsbA and DsbC was assayed. No clear conclusion could be drawn. Determining the order of disulfide bond interaction will inform the larger conversation on protein folding. The second experiment should be repeated in order to fully probe the order of interaction of Skp and DsbC.

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